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Direct and indirect uptake of pharmaceutical residues in a marine trophic segment

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Abstract

The ecological and economic value of the estuaries is undeniable, albeit these ecosystems continuously receive contaminated waters, loaded with pharmaceutical residues. One of the most prescribed pharmaceuticals is the antidepressant fluoxetine, and therefore is one of the most frequently detected pharmaceuticals in estuaries, that can induce severe effects on non-target species, potentially interfering with key functions, such as neural, behavioural and physiological processes. This study aimed to assess the ecotoxicological effects, as well as the differences resulting from direct and indirect exposure to the antidepressant fluoxetine, under estuarine conditions. For this, direct exposures to the pharmaceutical fluoxetine, at ecologically relevant concentrations, 0.3 μg L^{-1}, 20 μg L^{-1} and 80 μg L^{-1} were made using a primary consumer, the white common prawn, *Palaemon serratus*, and a secondary consumer, the green crab, *Carcinus maenas*. Additionally, to evaluate the toxicological effects of indirect exposure to fluoxetine, each upper trophic level species was fed with fluoxetine pre-exposed organisms from lower trophic levels. Finally, several biomarkers were determined: lipid peroxidation (LPO), DNA damage (DNAd), superoxide dismutase (SOD), catalase (CAT), Phase II glutathione S-transferase (GST) and acetylcholinesterase (AChE). *P. serratus* evidenced higher sensitivity to fluoxetine, and more specifically the direct exposure appears to induce more deleterious effects on *P. serratus*. The increase of CAT activity suggests that fluoxetine overwhelmed the organism's first antioxidant defences, resulting in damaging effects on DNA and increasing LPO levels. Higher oxidative stress was also observed in the direct exposure trial of *C. maenas* to fluoxetine, on which CAT activity had a significant decrease in both low and high treatments, with the opposite trend observed for LPO levels, suggesting a possible hormetic response and a failure in the antioxidant defence system. Additionally, in the present study, no locomotion inhibition nor behaviour effects were observed for *C. maenas*. Considering the application of the tested biomarkers as potential descriptors for the evaluation of *P. serratus* and *C. maenas* exposure to fluoxetine, these appear to be efficient biomarkers of the exposure type, highlighting the differences between the exposure trials here reported. Overall, this study demonstrated that direct exposure to fluoxetine contributes to a higher level of oxidative stress on both species.

Keywords: Fluoxetine; Biomarkers; Invertebrates; Ecotoxicology; Estuary

Resumo

O valor ecológico e económico dos estuários é inegável, bem como é incontestável as contínuas descargas de águas contaminadas nestas áreas, muitas vezes repletas de resíduos farmacêuticos. Um dos fármacos mais prescritos atualmente é o antidepressivo fluoxetina, e é consequentemente um dos fármacos mais frequentemente detetado em estuários. Por essa razão, adquire um grande potencial para induzir efeitos nefastos em espécies não alvo, interferindo em funções essenciais dos organismos, tais como as funções neurais, comportamentais e fisiológicas. Este estudo teve como objetivo avaliar os efeitos ecotoxicológicos resultantes de exposição direta e indireta ao antidepressivo fluoxetina, sob condições estuarinas. Para tal, exposições diretas ao fármaco fluoxetina, em concentrações ecologicamente relevantes, 0,3 μg L^{-1}, 20 μg L^{-1}e 80 μg L^{-1} foram realizadas utilizando para o efeito um consumidor primário, o camarão branco legítimo, *Palaemon serratus*, e um consumidor secundário, o caranguejo verde, *Carcinus maenas*. Além disso, para avaliar os efeitos toxicológicos da exposição indireta à fluoxetina, cada espécie do nível trófico superior foi alimentada com organismos pré-expostos à fluoxetina dos níveis tróficos anteriores. Finalmente, vários biomarcadores foram determinados, entre

os quais: peroxidação lipídica (LPO), dano no DNA (DNAd), superóxido dismutase (SOD), catalase (CAT), glutationa S-transferase (GST) e acetilcolinesterase (AChE). A espécie *P. serratus* demonstrou uma maior sensibilidade à fluoxetina. Particularmente, a exposição direta ao fármaco parece induzir efeitos mais deletérios nos organismos da espécie *P. serratus*. O aumento da atividade da CAT sugere que a fluoxetina superou as primeiras defesas antioxidantes do organismo, inclusive resultando em efeitos adversos no DNA, e aumentando os níveis de LPO. Maior stress oxidativo parece ocorrer também na exposição direta da espécie *C. amenas* à fluoxetina, onde a atividade da CAT teve uma diminuição significativa nas concentrações, mais baixa e mais alta, evidenciando uma possível resposta hormética. Com a mesma semelhança padrão, o inverso ocorreu nos níveis de LPO para os mesmos tratamentos, indicando uma potencial falha no sistema de defesa antioxidante do organismo. Além disso, no presente estudo, nenhuma inibição na locomoção, nem efeitos comportamentais foram observados em *C. maenas*. Considerando a aplicação dos biomarcadores testados como potenciais descritores para a avaliação da exposição das espécies *P. serratus* e *C. maenas* à fluoxetina, estes parecem evidenciar a sua eficácia ao tipo de exposição, destacando as diferenças entre os ensaios de exposição reportados neste estudo. No geral, este estudo demostrou que a exposição direta da fluoxetina na água contribui para níveis mais elevados de stress oxidativo, em ambas as espécies.

Palavras-chave: Fluoxetina; Biomarcadores; Invertebrados; Ecotoxicologia; Estuário

Resumo alargado

A presença generalizada de resíduos farmacêuticos em descargas de efluentes no ambiente, e particularmente em zonas estuarinas tem vindo a aumentar a preocupação do seu potencial efeito ecotoxicológico no ambiente, terrestre e aquático, e particularmente, nas espécies aquáticas endógenas. Estes compostos são continuamente descarregados no meio aquático, nomeadamente nas zonas estuarinas, causando a sua persistente presença nos ecossistemas aquáticos e produzindo efeitos adversos. A deteção dos resíduos farmacêuticos nestes ambientes aquáticos continua a crescer, atingindo concentrações de deteção entre os ng/L e mg/L em águas superficiais. Também, sendo considerados compostos biologicamente ativos, o seu modo de ação visavias metabólicas específicas, provocando efeitos adversos ao longo de toda a cadeia trófica, mesmo em concentrações ambientais baixas. No geral, o aumento da investigação científica e consequentemente, o aumento da literatura tem contribuído para uma melhor compreensão da forma como estes compostos farmacêuticos ocorrem nos ambientes costeiros e marinhos, e qual o seu destino e efeito ecotoxicológico. No entanto, poucos estudos são reportados no que diz respeito aos efeitos ecotoxicológicos de resíduos farmacêuticos em organismos aquáticos, particularmente marinhos ou estuarinos.

Os inibidores seletivos de recaptação de serotonina (SSRI- Selective serotonin reuptake inhibitors) são uma classe de antidepressivos amplamente utilizada, prescrita para o tratamento de ansiedade e depressão, por exemplo. Estes inibidores atuam bloqueando a reabsorção de serotonina nos neurónios, e subsequentemente aumentam os níveis de serotonina na fenda sináptica, maximizando a sua atuação nos nervos pós-sinápticos. A serotonina é importante num elevado número de funções biológicas, incluindo respostas de imunidade e comportamentais, tanto em vertebrados como em invertebrados. A fluoxetina, um dos antidepressivos mais prescritos globalmente, encontra-se em concentrações no meio aquático entre ng/L e µg/L, e por isso adquire uma particular atenção pelos seus potenciais efeitos de toxicidade aguda. No entanto, para compreender totalmente o efeito adverso dos fármacos, é necessário avaliar a rota de exposição ao longo da cadeia trófica. Para tal, compostos individuais como a fluoxetina, que vêm aumentando o risco adverso no ambiente necessitam de ser

monitorizados através de uma eficaz avaliação de risco ambiental, considerando particularmente os impactos na qualidade da água e na cadeia trófica, a nível bioquímico e fisiológico.

Os biomarcadores atuam como um instrumento de avaliação da qualidade ambiental, dando a conhecer as formas de atuação e respetivas respostas dos organismos a xenobióticos (compostos alheios aos ecossistemas), integrando as condições ambientais. Um biomarcador é definido como qualquer entidade biológica ou a resposta a um agente químico ou qualquer outro xenobiótico, que provoca uma alteração a nível bioquímico e fisiológico do organismo, e que pode ser quantificado. A avaliação das variações observadas em biomarcadores em estudos ecotoxicológicos, fornecem geralmente informação sobre a primeira exposição ao xenobiótico. Deste modo, são considerados sinais de alerta a curto prazo de potenciais efeitos adversos que podem causar danos significativos, mas também a longo prazo, podendo antever danos em níveis mais elevados de organização biológica. Os biomarcadores podem ser divididos de acordo com as suas características de atuação em biomarcadores de efeito, de exposição e de suscetibilidade. A exposição dos organismos a compostos xenobióticos pode resultar no aumento do stress oxidativo que leva à ocorrência de danos ao nível celular, nomeadamente a peroxidação lipídica e danos no DNA. No entanto, os mecanismos de defesa das células desempenham um papel fundamental na prevenção e minimização dos efeitos de stress oxidativo, entre os quais são exemplos as enzimas antioxidantes, como a catalase e a superóxido dismutase, e a enzima de biotransformação glutationa-S-transferase. Adicionalmente, a enzima acetilcolinesterase desempenha um papel fundamental nas funções motoras e neurológicas e é utilizada como indicador de neurotoxicidade.

Este estudo tem por base validar os resultados obtidos no âmbito de uma avaliação do quadro da qualidade ambiental, proporcionando uma visão complementar da saúde do ecossistema do estuário do Tejo. Desta forma, o presente estudo avaliará diversos biomarcadores, individualmente e de acordo com uma abordagem multivariada, para entender o modo de atuação do antidepressivo fluoxetina em organismos do estuário do Tejo, ao longo de um segmento trófico. Adicionalmente, serão avaliadas as diferenças resultantes de uma exposição direta e indireta, à toxicidade da fluoxetina. Para a exposição direta, organismos de níveis tróficos diferentes, um consumidor primário, o camarão branco legítimo, *Palaemon serratus*, e um consumidor secundário, o caranguejo verde, *Carcinus maenas*, serão expostos a água contaminada com fluoxetina em concentrações alvo (0,3 µg L^{-1}, 20 µg L^{-1} e 80 µg L^{-1}), enquanto que para a exposição indireta, os organismos do nível trófico anterior, previamente contaminados com diferentes concentrações de fluoxetina, servirão de alimento para os organismos do nível trófico superior. Finalmente, vários biomarcadores serão determinados, entre os quais, os níveis de peroxidação lipídica (LPO) e dano no DNA (DNAd), e as atividades das enzimas superóxido dismutase (SOD), catalase (CAT), glutationa S-transferase (GST) e acetilcolinesterase (AChE).

Os resultados obtidos evidenciaram que os organismos da espécie *P. serratus* foram mais sensíveis à exposição ao antidepressivo fluoxetina. A exposição direta à fluoxetina mostrou que as respostas dos biomarcadores obtiveram maiores atividades enzimáticas comparativamente à exposição indireta. O aumento da atividade enzimática da CAT, na exposição direta, pode indicar que a fluoxetina sobrecarregou a primeira linha de defesa antioxidante dos organismos, podendo ter resultado em valores mais elevados de LPO e DNAd, e consequentemente contribuindo para danos oxidativos nos organismos. Adicionalmente, os valores da atividade da enzima AChE, evidenciaram um possível aumento da toxicidade da fluoxetina ao nível neurológico e motor dos organismos da espécie *P. serratus*.

Relativamente à espécie do nível trófico superior, *C. maenas*, as respostas dos biomarcadores foram mais inconclusivas, não evidenciando diferenciação entre os biomarcadores, para ambas as exposições. No entanto, na exposição direta, os valores da enzima CAT, bem como, os níveis de LPO indicaram uma falha no sistema de defesa antioxidante à presença da fluoxetina, promovendo um aumento da produção de ROS nos organismos. A exposição indireta ao fármaco, evidenciou uma correlação negativa nos valores das enzimas GST e SOD, sugerindo que a capacidade antioxidante da

enzima SOD não foi capaz de suprimir e catalisar a quantidade de radicais superóxido, e desta forma foi induzida a enzima GST com o intuito de resistir à presença da fluoxetina nos organismos. Relativamente aos efeitos da fluoxetina na locomoção e atividade neurológica dos organismos *C. maenas*, o presente estudo evidenciou que não existiu inibição da locomoção nem efeitos adversos comportamentais e neurológicos, corroborados pelos valores da enzima AChE.

No geral, o estudo demonstrou que a espécie *P. serratus* apresenta uma maior suscetibilidade ao antidepressivo fluoxetina, e que a espécie *C. maenas* aparenta exibir uma maior resistência a este fármaco nas concentrações alvo utilizadas, tanto exposta diretamente ao fármaco bem como via alimentação.

O conhecimento sobre a contaminação por fármacos e os seus efeitos biológicos nos níveis tróficos superiores é essencial para lidar com os impactos dos resíduos farmacêuticos dentro de um quadro ecológico ambiental. De acordo com a literatura, existem poucos estudos sobre os efeitos do antidepressivo fluoxetina nas espécies *P. serratus* e *C. maenas*, portanto nesse sentido, o presente estudo oferece conhecimento adicional neste campo. Tanto quanto é do nosso conhecimento, não existem outros estudos que relatem os efeitos da exposição indireta a um fármaco que simule a contaminação por fluoxetina que ocorre ao longo de um segmento trófico do estuário do Tejo.

Apesar dos resultados obtidos, diversas formas de complementar este estudo passam pela realização de ensaios de bioacumulação e bioconcentração, que são uma chave fundamental para identificar e perceber o modo de atuação do antidepressivo fluoxetina nos organismos. Adicionalmente, a análise química da água também pode ser um caminho para entender os efeitos da potencial toxicidade da bioacumulação da fluoxetina e como esta afeta os organismos.

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Abbreviations and Symbols

AChE	Acetylcholinesterase
BAF	Bioaccumulation factor
BHT	Butylated hydroxytoluene
CAT	Catalase
CDNB	Dinitrochlorobenzene
DNAd	DNA damage
DTNB	Nitrobenzoic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GSH	Oxidized glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
K_2HPO_4	Monobasic potassium phosphate
KCl	Potassium chloride
KH_2PO_4	Dibasic potassium phosphate
LPO	Lipid peroxidation
N_2	Nitrogen
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
PAR	Photosynthetically active radiation
PCBs	Polychlorinated biphenyls
PMSF	Phenylmethylsulfonyl fluoride
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate detergent
SOD	Superoxide dismutase
SSRI	Selective serotonin reuptake inhibitors
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid

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1. Introduction

Over the last decades, the increasing economic and social expansion of human activities in coastal areas, mainly as a result of the unbridled growth of industrialization and coastal settlement, has led to significant modifications in the environment, with water pollution representing a major issue for coastal organisms. (e.g. Kennish, 2002; Vasconcelos *et al*., 2007; Vikas and Dwarakish, 2015).

According to Crain *et al.* (2009), human pollution has been changing marine habitats, transforming continuously physical and chemical properties, and ultimately leading to its permanent loss. Additionally, these marine ecosystems are frequently suffering from contaminated effluents discharges.

Estuaries are considered valuable ecosystems, with high ecological and economic value (Beck *et al*., 2001; Costanza *et al*., 1997). Still, these areas are prone to receiving contaminated waters from direct discharges and indirectly from river inputs, containing a wide range of potential pollutants from the well-known contaminants such as metals (e.g. copper, zinc) (Fonseca *et al*., 2011, 2015) and Polychlorinated Biphenyls (PCBs) (Carpenter, 2006), to the newly emerging contaminants, pharmaceutical residues (such as antibiotics and antidepressants) (Fabbri and Franzellitti, 2016). The continuous release of these emerging contaminants to the aquatic environment led to recognition from the European governmental entities, for the need to improve the legislation regarding the management of the risk and spreading of these pharmaceutical residues (2013/39/EU; EU Commission Implementing Decision 2018/840). Moreover, government agencies have released guidelines on how pharmaceuticals should be evaluated in environmental compartments, and the potential ecological risks assessed (Hagger *et al*., 2008; Sanchez and Porcher, 2009).

Pharmaceuticals are a class of emerging environmental contaminants that originate from human and veterinary medicine (Fent *et al*., 2006). These chemicals are biologically active compounds, designed to have a specific mode of action, which may cause toxicity in non-target organisms chronically exposed in the environment (Claessens *et al.,* 2013; Miller *et al.*, 2015). This pharmaceuticals are known to occur widely in the environment of countries considered industrialized, and coastal zones such as estuaries are common sites for pharmaceuticals' discharges (Gaw *et al*., 2014; Silva *et al*., 2014). Over the last decades, the improvement in medicine has contributed to the rising number of new pharmaceuticals (David *et al*., 2015; Scherer, 2000), and subsequently, a diverse number of these compounds are finding their way into marine ecosystems (Reis-Santos *et al*., 2018), in environmental concentrations generally range from ng/L up to a µg/L, yet mg/L. Pharmaceuticals residues have been frequently detected in wastewater, freshwater and coastal environments worldwide (e.g. aus der Beek *et al*., 2016; Daughton, 2016; Kümmerer, 2004), and therefore, understanding the occurrence and toxicity effects of these pharmaceuticals in the environment is urgent since its use has been increasingly growing worldwide (Küster and Adler, 2014). Many compounds appear to be relatively persistent in the aquatic environment and the current studies only express a slight portion about the ecotoxicological effects of pharmaceuticals on aquatic organisms (aus der Beek *et al*., 2016; Christen *et al*., 2010). Concerning the marine environment, the literature is still lacking, and just recently the research focusing on the potential effects, fate and ecotoxicology of pharmaceuticals on these ecosystems has grown (Fabbri and Franzellitti, 2016; Gaw *et al*., 2014; Klosterhaus *et al*., 2013). Alongside, Daughton (2016) presented a review of the exponential growth of literature regarding pharmaceutical contamination in the environment.

From all the pharmaceutical classes, antidepressants appear to be one of the most frequently detected compounds in the aquatic environment with concentrations ranging from ng/L to µg/L (aus der Beek *et al*., 2016; Silva *et al*., 2017), likely since these are one of the most prescribed drugs in human medicine. The introduction of these antidepressants in the ecosystem generally exceeds the capacity to

metabolize and excrete them, and are thus considered to be persistence or pseudo-persistent *(*Arnold *et al.*, 2014). On the marine environment reported concentrations were detected in both invertebrates and vertebrates, with concentration ranges up to 600 ng/g in vertebrates and up to 320 ng/g in invertebrates (Miller *et al.*, 2018). Furthermore, antidepressants can cause severe changes in the organisms' reproduction cycles, growth and behaviour (e.g. Ford and Fong, 2016; Henry *et al*., 2004; Martin *et al*., 2017; Yang *et al*., 2014).

Biomonitoring this type of pharmaceuticals in aquatic biota is sparse, but Minguez *et al*. (2016) shown studies related to the measurement of toxicity of 48 pharmaceuticals in aquatic wildlife reporting some compounds that exhibited strong toxicities even at low concentrations (e.g. antibiotics, antidepressants and antifungals). Nevertheless, to fully understand the hazardous effect of pharmaceuticals it is necessary to assess the route of exposure of the organisms itself, but also the exposure route within the trophic chain (Miller *et al*., 2018).

According to Meador (2006), the term bioaccumulation is defined as the uptake and subsequent accumulation of substances from all the surrounding environment sources, such as water, food and sediment, and the bioaccumulation factor (BAF) can be estimated from the ratio of the chemical concentration within the organism with the concentration detected in the ecosystem (Arnot and Gobas, 2006), whereas the term bioconcentration is a specific bioaccumulation process, where aquatic organisms accumulate a substance directly from the water (Zenker *et al*., 2014), and the bioconcentration factor (BCF) is described by Arnot and Gobas (2006), as the ratio of the chemical concentration in the internal tissues of the organism and the exposure concentration. The BCF is believed to be more consistent when it is determined in laboratory exposures, where the concentrations are well known, thus information on bioaccumulation is more common than on bioconcentration. Bioaccumulation and bioconcentration factors are essential to assess the ecotoxicological risk of antidepressants (e.g. Ding *et al*., 2017; Gray, 2002; Puckowski *et al*., 2016).

Pharmaceuticals bioaccumulation reports are well known regarding antibiotics and antidepressants (Puckowski *et al*., 2016), being antidepressants of special interest, since they are one of the most prescribed medicines for treat depressions and anxiety disorders (Lindsley, 2012). Antidepressants are frequently detected in aquatic environments (Fonseca *et al*., 2020; Reis-Santos *et al*., 2018) and entail significant effects both in vertebrate and invertebrate species. Moreover, Martin *et al*. (2017) and Yang *et al.* (2014) reported that antidepressants can cause irreversible changings in the organisms' growth and behaviour, while Henry *et al.* (2004) demonstrated that the reproduction cycles from the daphniidae *Ceriodaphnia dubia* decrease significantly.

Regarding the trophic transfer data, it should be a matter of concern for ecological risk assessment of pharmaceutical substances in aquatic food webs. Although most of the antidepressants appear to have the potential to accumulate and magnify, there is a shortage on the evidence of trophic transfer (e.g. Heynen *et al*., 2016; Xie *et al*., 2017).

Selective serotonin reuptake inhibitors (SSRI) are a widely used type of antidepressant, prescribed for the treatment of anxiety and depression disorders. SSRI acts blocking the reabsorption of serotonin into neurons, and subsequently increasing the levels of serotonin in the cleft nerve (Beasley *et al*., 1992). Fluoxetine, an antidepressant defined as SSRI, is one of the most prescribed antidepressants nowadays and acts inhibiting the reuptake of serotonin transporter protein, located in the presynaptic terminal (Brooks *et al*., 2003; Beasley *et al*., 1992). According to Benfield *et al*. (1986), fluoxetine can facilitate serotoninergic neurotransmission through inhibition of neuronal reuptake of serotonin. It is known that serotonin is present both in vertebrates and invertebrates, being involved in the physiological and behavioural functions of these organisms (e.g. Robert *et al*., 2016). Serotonin controls a wide-range of systems, and changes in serotonin levels may alter fish behaviour (e.g. Saaristo *et al*., 2017), and also disrupt growth and reproduction in invertebrates (e.g. Paterson and Metcalfe, 2008; Silva *et al*., 2016). The concentration ranges that fluoxetine is found in the environment differ

from ng/L to µg/L (Duarte *et al.*, 2019), and so the potential and acute toxicity effects related to this antidepressant deserve particular attention (Brooks *et al.*, 2003). From marine producers to primary and secondary consumers, fluoxetine affects behaviour and endocrine and reproductive processes even at a short time frame exposure (e.g. Ding *et al*., 2017; Duarte *et al.*, 2019; Fong and Ford, 2014; Silva *et al*., 2016; Sumpter *et al*., 2014). Additionally, Duarte *et al.* (2019,2020), Silva *et al*. (2016) and Mesquita *et al.* (2011) have described the fluoxetine toxicity in the aquatic environment, in particular in fish, molluscs and crustaceans. According to Robert *et al*. (2016), fluoxetine can disrupt neuroendocrine control in crustaceans, as they interfere with the normal regulation of the serotonergic system. Aquatic organisms are particularly important targets, as they are exposed via wastewater residues over their whole life (Fent *et al*., 2006). Moreover, fluoxetine has been shown to accumulate both in the organism as well as on the environment itself (e.g. Ding *et al*., 2017; Fong and Ford, 2014; Puckowski *et al*., 2016). Though the mode of action of fluoxetine is already described in the literature (Brooks *et al*., 2003), its potential to bioaccumulate via direct and indirect uptake is unknown, as well as its effects along the trophic chain.

Since individual compounds such as fluoxetine have increased risk in the environment (Caldwell *et al*., 2014; Fong and Ford, 2014), the need to have an efficient ecosystem monitoring, considering water quality and biological impacts acquired a new significance.

According to Van Gestel and Van Brummelen (1996), a biomarker is any biological entity or response to a chemical agent, considered at the sub-individual level, measurable or its sub-products, within the organism. Biomarkers provide insights on the first response to chemical exposures and of the contamination effects at the sub-individual level, at short and long term exposures, thus, can be considered early warning signals of potential adverse effects that may cause significant damage effects, later in time at higher levels of biological organization (e.g. tissue, organ, individual) (der Oost *et al*., 2003). They can also be used as a tool for detection of simultaneous exposure to various chemicals, or for the identification of toxicity mechanisms (Timbrell, 1998). Biomarkers are divided, according to its own characteristics, into biomarkers of effects, that are defined as quantifiable changes that an individual endures and that indicates exposure to a compound that produce deleterious effects at the cellular level (Timbrell, 1998), biomarkers of exposure, that reflect biochemical behaviours that can be measured in the organism or after xenobiotic excretion and are used to determine different characteristics of an organism exposure (Timbrell, 1998) and biomarkers of susceptibility that indicates the natural characteristics of an individual that make it more susceptible to the effects of exposure to a chemical (Broeg and Lehtonen, 2006; Timbrell, 1998).

The exposure of organisms to any toxic molecule, antidepressants included, may induce oxidative stress, which can lead to damage at a cellular level, such as genetic material oxidation (DNAd) as well as promoting lipid peroxidation (LPO). However, there are defence mechanisms that allow organisms to minimize these oxidative stress effects, and therefore, play a primary role in its prevention. Antioxidant enzymes (e.g. SOD, CAT) as well as biotransformation enzymes (e.g. GST), are frequently used as biomarkers in ecotoxicological assays since they are considered good indicators of exposure to environmental contaminants. Also, cholinesterases enzymes (e.g. AChE) have an essential role in neuronal and motor functions of the individuals and are considered to be a good bioindicator of neurotoxicity.

The continued released of pharmaceuticals into the aquatic environment can lead to disturbances on the aquatic organisms and contribute to behavioural and physiological changes in species along the trophic chain (Zenker *et al*., 2014). Combining biomarker responses with the assessment of bioaccumulation and potential trophic transfer of pharmaceuticals can lead to a better understanding of its action in multiple organisms, and ultimately of its potential impacts in the environment (Liu *et al.*, 2017), hence, a multi-taxa approach, as well as a multi-biomarker approach,

can provide a more integrative response to those environmental changes and provide further insight of the ecosystem pressures (Fonseca *et al.*, 2011; Richardson *et al*., 2011).

Aiming to validate the results within a comprehensive assessment of environmental quality framework, providing a complementary view of the Tejo estuary ecosystem health, in the present study will be assessed several biomarkers, individually and combined in a multi-biomarker approach in an organism from several trophic levels. Additionally, the toxicity exposure route of the pharmaceuticals' residues will be evaluated to disentangle how it is modulated under direct and indirect exposure (via feeding). For this, direct exposures to the pharmaceutical substance fluoxetine, at ecologically relevant concentrations, 0.3 μg L^{-1}, 20 μg L^{-1} and 80 μg L^{-1} will be made using several species from the Tejo estuary in order to have a representation of the different trophic levels, from primary consumers and producers to higher predators: a primary producer, the diatom *Phaeodactylum tricornutum* (Cabrita *et al*., 2017; Duarte *et al*., 2019), usually found in estuarine and coastal zones, and considered to be tolerant to a numerous stressors effects, being suitable as a good bioindicator in several assays related to its ability to resist environmental stress, a primary consumer, the white common prawn *Palaemon serratus*, which is a very common species in the coastal zones with a wide-range geographical distribution that can be found on rocky shores, within estuaries and in deeper offshore waters (Haig *et al*., 2014), and a secondary consumer, the green crab *Carcinus maenas*, that lives mostly in permanent contact with the sediment and is considered to be sensitive to a wide range of aquatic contaminants (Duarte *et al*., 2017; Rodrigues and Pardal, 2014), all under estuarine conditions (temperature, salinity and pH). Overall, all species considered in this study are common in Portuguese estuaries (Fonseca *et al*., 2011; Gomes *et al*., 2013), and play an important ecological role in the community, and are considered suitable bioindicators of habitat quality that have been used in several ecotoxicological studies (e.g. Cabrita *et al*., 2017; Haig *et al*., 2014; Rodrigues and Pardal, 2014). Additionally, the present study intends to evaluate the toxicological effects of indirect exposure to this pharmaceutical molecule, by feeding each upper trophic level with fluoxetine pre-exposed organisms from lower trophic levels, under the same target concentrations. Ecotoxicity of the different exposure trials will be addressed using ecotoxicological biomarkers. Furthermore, for each species, several biomarkers were determined: lipid peroxidation (LPO), DNA damage (DNAd), superoxide dismutase (SOD), catalase (CAT), Phase II glutathione S-transferase (GST) and acetylcholinesterase (AChE). LPO and DNAd are linked to contaminants exposure that produces deleterious effects at the cellular level, while the activity levels of the antioxidant enzymes SOD and CAT are involved in the reduction of oxidative stress and the detoxification of reactive oxygen species (ROS). Moreover, GST is involved in enzymatic biotransformation activity, performing the metabolization of xenobiotics or their metabolites, facilitating their excretion, whilst AChE activity is an indicator of neurotoxicity that plays an important role in neuronal and motor functions.

2. Materials and Methods

2.1. Study area and sample collection and maintenance

The sampling area was chosen according to previous works focusing the Tejo estuary (Vasconcelos *et al*., 2007), in particular the Alcochete salt marsh and the surrounding water bodies where species were capture due to its low contamination levels and almost pristine condition, but also due to the simultaneous presence of all the target species (Duarte *et al*., 2013). All organisms were captured and transported to the laboratory in refrigerated and aerated containers.

Phaedactylum tricornutum was grown in the laboratory using F/2 medium, at 18 ºC under a photosynthetically active radiation (PAR) intensity of 200 μmol photons m^{-2} s^{-1} using a 16h/8h (light/dark) photoperiod.

Individuals from *Palaemon serratus* were brought to the laboratory and selected according to their size and placed in acclimation conditions resembling environmental salinity and water temperature, using artificial seawater to minimize field contamination.

Regarding *Carcinus maenas* individuals, were brought to the laboratory where males and females were separated. Males were weighed and measured length before acclimatization, to select similar individuals for the exposure trials, and to exclude any potential effects of gender. The exposure conditions were set as above mentioned for *P. serratus*.

2.2. Exposure trials

a) Phaeodactylum tricornutum

Phaeodactylum tricornutum was grown for 48 hours, after which it was exposed to fluoxetine target concentrations (0 μg L^{-1}, 0.3 μg L^{-1}, 20 μg L^{-1} and 80 μg L^{-1}). The diatom cultures were exposed to these conditions for an additional 48 h period to incorporate this contaminant. At the end of the exposure period, culture samples were centrifuged at 4000 rpm for 15 min at 4 ºC and the supernatant discarded. As fluoxetine impairs *P. tricornutum* growth, the volume of pellet centrifuged was adjusted in each control treatment to produce pellets with the same number of cells, independently of the fluoxetine exposure. Pellets were flash-frozen in liquid N_2 and stored at -80 ºC. The same procedure was applied to a culture without exposure to fluoxetine. This experiment was not used to evaluate toxicity effects in *P. tricornutum* as this was performed in a previous work (Feijão *et al.*, 2020), and thus this exposure aimed only to generate contaminated and non-contaminated pellets to feed the next trophic level.

b) Palaemon serratus

Palaemon serratus individuals were exposed to 4 treatments: control, low, medium and high fluoxetine concentrations (0 μg L^{-1}, 0.3 μg L^{-1}, 20 μg L^{-1} and 80 μg L^{-1}). Four replicates mesocosms units, with 6 individuals each, were used per treatment to ensure representativeness and to avoid mesocosms exposure artefacts. Both direct and indirect exposure trials were performed for 7 days, on which water samples for chemical analysis were collected. During the direct exposure trial, water was contaminated with fluoxetine at target concentrations, while in the indirect exposure trial *P. serratus* individuals were fed with fluoxetine pre-exposed *P. tricornutum*, as described above (section 2.2a). Water from the mesocosms was exchanged every 2 days and water samples collected for fluoxetine quantification.

At the end of the exposure trials, individuals were sacrificed and dissected in a cold-block. 200 mg of tissue samples (abdomen and cephalothorax) were stored at -80 ºC until analysis. Biomarkers Lipid Peroxidation and DNA damage were evaluated on *P. serratus* abdomen, while Acetylcholinesterase, Superoxide dismutase, Catalase and Glutathione S-transferase enzymatic activities were tested using *P. serratus* cephalothorax. Regarding the bioconcentration analysis, the necessary individuals up to 1 g (whole-body) were collected from each treatment and stored at -80 ºC until analysis.

c) Carcinus maenas

Carcinus maenas individuals were exposed to 4 treatments, control, low, medium and high (0 μg L^{-1}, 0.3 μg L^{-1}, 20 μg L^{-1} and 80 μg L^{-1}) fluoxetine concentration. Four replicates mesocosms units, with 6 individuals each, were used *per* treatment to ensure representativeness as above-mentioned (section 2.2b). Both direct and indirect exposure trials last for 7 days. Water for chemical analysis was also collected, on the first, third and seventh day. During the direct exposure trial, water was

contaminated with fluoxetine at target concentrations, while during the indirect exposure trial *C. maenas* were fed with *P. serratus* previously injected with fluoxetine doses similar to those applied during the direct exposure trials. Injected volume and concentration were determined according to *P. serratus* weight to have similar fluoxetine concentrations on a wet weight basis.

At the end of the exposure trials, individuals were sacrificed at -20 ºC and dissected in a cold-block. Hepatopancreas and muscle samples were stored for biomarker analysis at -80 ºC. Lipid peroxidation, DNA damage, Superoxide Dismutase, Catalase and Phase II Glutathione S-transferase activities were evaluated on *C. maenas* hepatopancreas, while Acetylcholinesterase was evaluated on *C. maenas* muscle. For bioconcentration analysis, were collected hepatopancreas samples up to 1 g from each individual and stored at -80 ºC until analysis.

2.3. Biomarkers quantification

The biomarkers analysed were Lipid peroxidation (LPO), DNA damage (DNAd), Catalase (CAT), superoxide dismutase (SOD), Glutathione S-transferase (GST) and acetylcholinesterase (AChE).

Replicate samples of the tissues collected were homogenised in 1:5 (w/v) of 100 mM monobasic potassium phosphate/dibasic potassium phosphate (K_2HPO_4/KH_2PO_4) buffer (pH 7.4) containing 0.15 M KCl (potassium chloride), 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM DTT (dithiothreitol) and 1 mM EDTA (ethylenediaminetetraacetic acid), to avoid protein oxidation and protease activity. After homogenization aliquots of 50 µL were collected for LPO and DNAd assays. Three microliters of BHT (butylated hydroxytoluene) (1:15 v/v sample) were added to LPO aliquots to prevent further lipid peroxidation until analysis. The remaining homogenates were centrifuged at 12000 x g for 20 minutes at 4 ºC and aliquoted 200 µL of the supernatant for each SOD, CAT and GST protocols.

For AChE quantification, the replicate samples were homogenised in 1.5 (w/v) of 100 mM monobasic potassium phosphate/dibasic potassium phosphate (K_2HPO_4/KH_2PO_4) buffer (7.2) containing 0.075 M acetylthiocholine, 10 mM DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] with 17.855 mM $NaHCO_3$ (sodium bicarbonate). Following sonication, the homogenate was centrifuged at 11000 x g for 3 minutes at 4 ºC and aliquoted 200 µL of the supernatant for AcHE protocols.

All extracts were also separated into additional aliquots for the analysis of protein content.

All biomarker responses were determined in a microplate reader (Biotek Synergy HT), and each reading was done in quadruplicate.

a) Lipid Peroxidation

Lipid peroxidation (LPO) was determined according to Ohkawa, *et al.*, (1979), in which the products of the degradation of polyunsaturated fatty acid peroxides of membrane lipids and aldehydes typically, react with 2-thiobarbituric acid (TBA) forming coloured malonaldehyde commonly known evaluated as thiobarbituric acid reactive substances (TBARS). The TBARS concentration was measured spectrophotometrically at 535 nm ($\varepsilon = 1.56 \times 105\ M^{-1}\ cm^{-1}$), after the reaction occurred in a final reaction mixture containing 60 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, TCA 12 % and TBA 0.73 %, at 97°C for 60 min. The reaction was stopped on ice and samples are centrifuged at 13,400 x g for 3 minutes. Lipid peroxidation was expressed as nmol of TBARS formed per mg of tissue wet weight.

b) DNA damage

To determine the DNA damage (DNAd) level (Olive, 1988), samples were analysed by DNA alkaline precipitation, adding 2 % SDS containing 10 mM EDTA, 10 mM Tris base (pH 12.4) and 50 mM NaOH. After 1 minute, 0.12 M KCl was gently added and the mixture is incubated at 60 °C for 10

min. Samples were then cooled on ice for 15 min and centrifuged at 8000 x g for 5 min (4 °C). The supernatant was removed, and the DNA concentration determined following the addition of Hoechst dye (1 μg mL^{-1} in 0.1 M K-phosphate buffer, pH 7.4). The fluorescence of the reaction product was determined at 360 and 460 nm excitation and emission wavelengths. Fluorescence values will be compared to a DNA standard curve and DNAd expressed as μg DNA per mg of wet weight.

c) Catalase

Catalase (CAT) activity, according to Aebi (1974), was measured by monitoring spectrophotometrically at 240 nm the consumption of its substrate, hydrogen peroxide (30 mM H_2O_2 in 50 mM phosphate buffer, pH 7). Catalase activity was calculated as the difference in the absorbance during the time course ($\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$) and expressed as μmol min^{-1} mg^{-1} protein.

d) Superoxide dismutase

Superoxide dismutase (SOD) activity was determined according to McCord and Fridovich (1969), combining the protein extract with 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 1.5 mM hypoxanthine, 0.15 mM cytochrome c and 30 mU mL^{-1} of xanthine oxidase. Enzymatic reduction of cytochrome c by the xanthine oxidase/hypoxanthine system was monitored spectrophotometrically at 550 nm. One unit of SOD is defined as the amount of enzyme that inhibits the reduction of cytochrome c by 50 %. Superoxide dismutase activity is expressed as U mg^{-1} of total protein concentration.

e) Phase II Glutathione S-transferase

Glutathione S-transferase (GST) activity was measured according to Habig *et al*. (1974), following the conjugation of the protein extract with oxidized glutathione (GSH) and CDNB (1-chloro-2,4-dinitrobenzene), in a final reaction mixture containing 100 mM phosphate buffer (pH 6.5), 20 mM CDNB and 20 mM reduced GSH. The change in absorbance was recorded spectrophotometrically at 340 nm, and the enzyme activity expressed as nmol CDNB conjugate formed per mg of total protein per minute of reaction ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$).

f) Acetylcholinesterase

The acetylcholinesterase (AChE) activity was determined according to Ellman *et al.* (1961) with modifications to microplate reader by Guilhermino *et al*., (1996). Briefly, the rate of production of thiocholine, as well as acetylthiocholine hydrolyzation, is measured by combining the protein extract with 0.2 ml acetylcholine,1 mL DTNB and 30 mL Phosphate buffer 0.1 M (pH 7.2). The continuous reaction of the thiol with 5,5'-dithio-bis-2-nitrobenzoate ion produces the yellow anion of 5-thio-2-nitro-benzoic acid. The rate of colour production was measured spectrophotometrically at 412 nm in a microplate reader ($\varepsilon = 13.6 \times 10^3\ mM^{-1}\ cm^{-1}$) for 10 minutes, at 20 seconds timesteps and the AChE activity expressed as nmol of substrate hydrolysed per min per mg of total protein.

g) Protein quantification

The protein content (in mg) was determined according to Bradford (1976), adapted to 96-wells microplates. 250 μL of Bradford solution (Sigma) was added to 1-10 μL of each replicate sample, then the absorbance was read spectrophotometrically at 595 nm after 15 min of incubation. Bovine serum albumin solution (1 mg mL^{-1}) was used as protein standard, and the protein concentration was expressed as mg per mL of solution.

3. Data analysis

Data normality and homogeneity of variances was tested using Shapiro-Wilk and Levene tests. When the two parametric assumptions were not verified, the non-parametric test Kruskal-Wallis was applied and the differences in biomarker responses among replicates tanks per treatment and biomarker responses among treatments were obtained according to the *post-hoc* Dunn test. After, a Mann-Whitney test was performed to identify the differences between the direct and indirect exposure for both *P. serratus* and *C. maenas*. To test for correlations between biomarker responses for both species and exposure concentration, was applied the Spearman rank-order correlation coefficient (r_s) analysis. Finally, a multivariate analysis approach was performed applying a CAP (Canonical Analysis of Principal coordinates). All analyses were performed in SPSS software (SPSS Statistical, 2016) and a significance level of 0.05 was considered for all statistical tests used.

4. Results

Regarding the mortality rates in all exposure trials for both species, was observed that for *P. serratus,* no individual died from neither direct nor indirect exposure trial. On the other hand, concerning *C. maenas*, only one individual from the high concentration tank died in the indirect exposure trial.

4.1. Palaemon serratus

Concerning biological responses from *P. serratus* to fluoxetine exposure, only the indirect exposure demonstrated significant differences in SOD activity after exposure, in which all treatments differed from control, that had the highest activity ($H = 16.69$, p-value < 0.05, Fig. 4.1-i). Significant differences were observed in CAT activity in both direct and indirect exposures experiments ($H = 15.44$, p-value < 0.05, $H = 20.94$, p-value < 0.05, respectively). In the direct exposure trials, all treatments differed from the control that had the lowest activity, whilst in the indirect exposure experiment only in the high dosage did CAT activity increased compared to the other treatments (Fig. 4.1-ii). Regarding GST activity, a significant difference was observed in the indirect exposure setup ($H = 8.07$, p-value < 0.05), in which the low and medium (at the threshold level of significance), that had the lowest activities, differed from control. Additionally, the low and high treatments differ between each other (Fig. 4.1-iii). Concerning AChE activity, significant differences were found in the direct exposure experiments ($H = 9.57$, p-value < 0.05, Fig. 4.1-iv), where all treatments differed from control, that had the highest activity. As for the biomarkers of effect, LPO and DNAd, only the DNA damage levels exhibited significant differences in the direct exposure trials ($H = 10.52$, p-value < 0.05, Fig. 4.1-v). The medium and high treatments, both with the highest levels, differed from control. Moreover, were found differences between low and high treatments.

Mann-Whitney test demonstrated significant differences for all biomarkers between both exposure trials, except for the two control treatment groups, where significant changes could not be detected. Regarding the antioxidant enzymes, SOD activity showed significant differences at medium and high treatments between the two exposure groups ($U = 6.00$, p-value < 0.05, $U = 29.00$, p-value < 0.05). On the other hand, CAT exhibited significant differences for low and medium treatments ($U = 28.00$, p-value < 0.05, $U = 20.00$, p-value < 0.05), when comparing direct and trophic exposure groups. GST activity revealed significant differences only at between the individuals from both exposure trials subjected to the low treatments ($U = 30.00$, p-value < 0.05), whereas AChE did not demonstrate significant differences in any of the considered doses applied. Concerning biomarkers of effect, both exhibited significant differences. For LPO, low, medium and high treatments have shown significant

differences between the two exposure groups (U =10.00, p-value < 0.05, U =12.00, p-value < 0.05, U =21.50, p-value < 0.05) , whilst DNAd presented significant differences only at medium and high treatments (U =8.00, p-value < 0.05, U =2.00, p-value < 0.05).

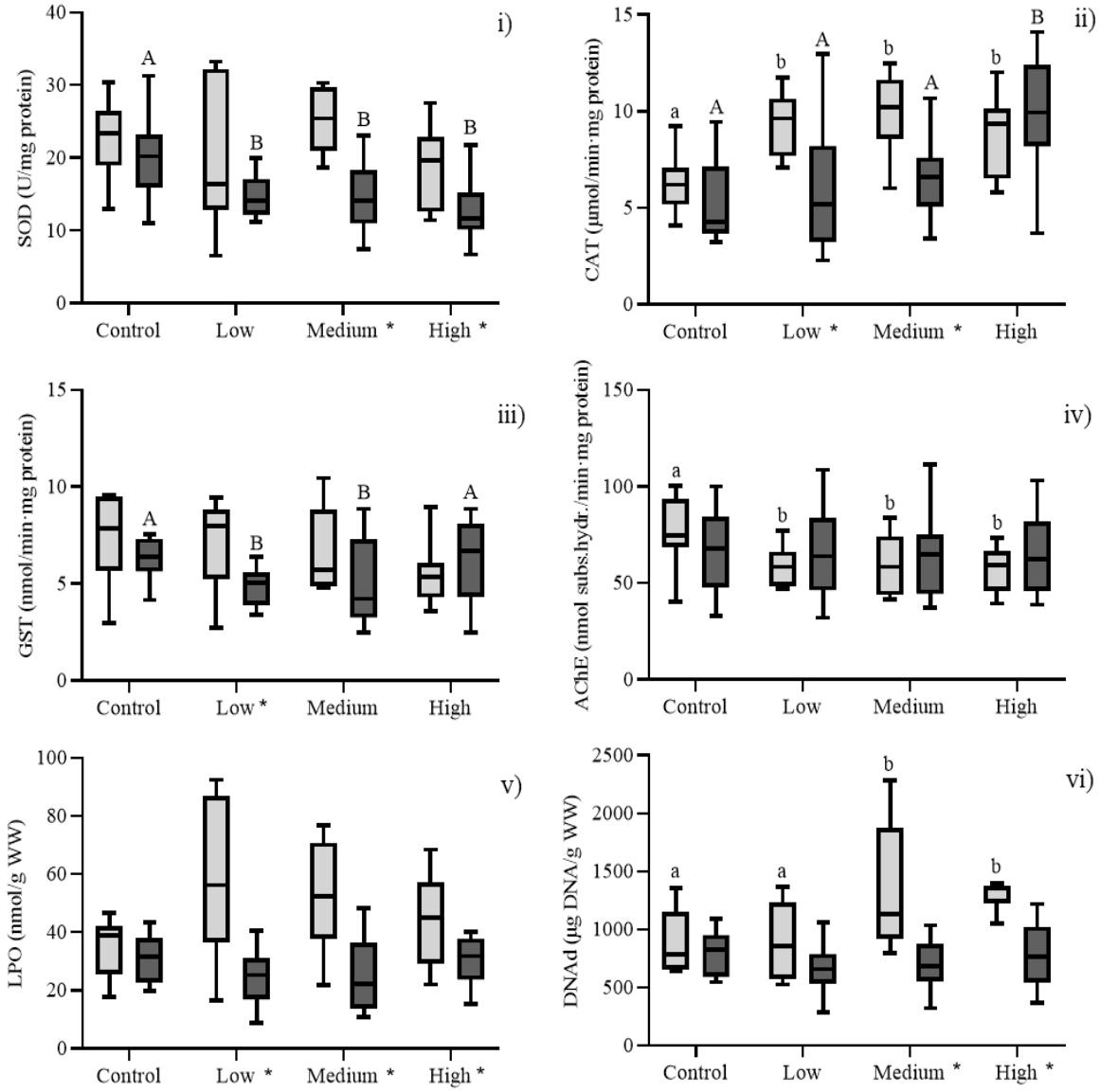

Fig. 4.1 - Biomarkers responses from *P. serratus* to direct (grey boxplots) and indirect exposure (black boxplots) of fluoxetine. i)- SOD activity response; ii)- CAT activity response; iii)- GST activity response; iv)- AChE activity response; v)- LPO level response; vi)- DNAd level response. Number of replicates for direct exposure: n=41 for CAT, SOD, GST and AChE; n=33 for LPO and DNAd. Number of replicates for indirect exposure: n=62 for SOD, CAT, GST and AChE; n=47 for LPO and DNAd. Boxplots represent median and whiskers represent minimum and maximum values; lowercase letters indicate significant differences at p-value < 0.05 for Kruskal-Wallis test for the direct exposure and uppercase letters indicate significant differences at p-value < 0.05 for Kruskal-Wallis test for the indirect exposure; asterisks denote significant differences among the same treatments on the direct and indirect exposure trials for Mann-Whitney test.

4.2. Carcinus maenas

Carcinus maenas biomarkers responses to the direct and indirect exposure to fluoxetine are displayed in Fig. 4.2. As for the antioxidant enzymes activities, only CAT exhibited significant differences at the direct exposure trials, with low and high treatments, that had the lowest activities, differing from control. Additionally, were found significant differences between low and medium treatments during the abovementioned exposure type. Furthermore, at the threshold level of significant,

high and medium treatments showed differences between each other (H =10.09, p-value < 0.05, Fig. 4.2-ii). Concerning GST, in the indirect exposure the high treatment, with the highest activity, was different from all others (H =13.29, p-value < 0.05, Fig. 4.2-iii). As for AChE, no significant differences were found. Regarding the biomarkers of effect, just LPO levels showed significant differences in the direct exposure trials, where low and high treatments differed from control. Also, low, that had the highest activity, differ from medium treatment (H =-13.71, p-value < 0.005, Fig. 4.2-v).

According to the Mann-Whitney test, significant differences between both exposure trials were only found concerning CAT and LPO biomarkers. CAT activity exhibited differences for low, medium and high treatments (U =47.00, p-value < 0.05, U =57.00, p-value < 0.05, U =42.00, p-value < 0.05), while LPO showed significant differences for low and medium treatments (U =8.00, p-value < 0.05, U =53.00, p-value < 0.05).

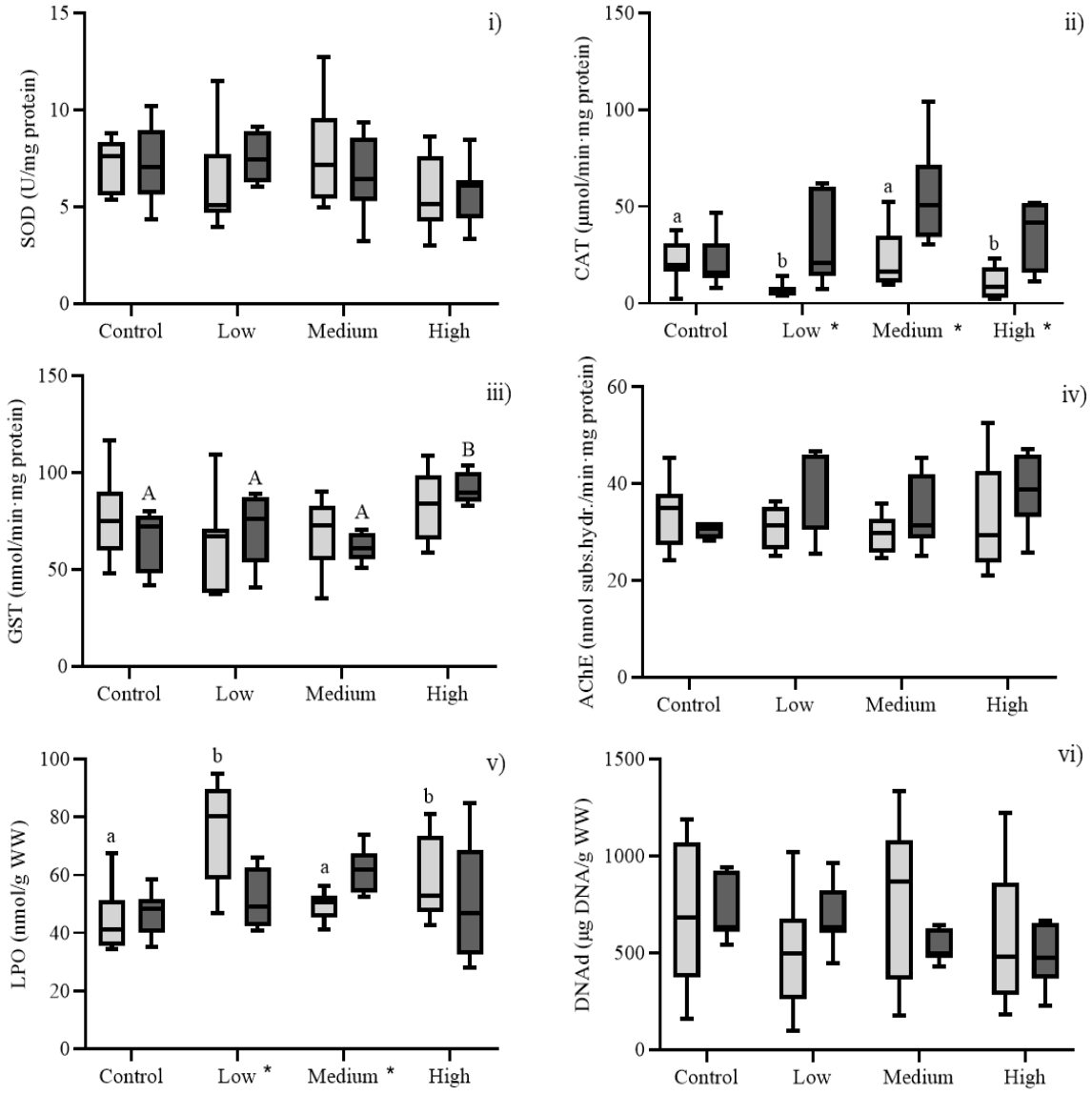

Fig. 4.2 - Biomarkers responses from *C. maenas* to direct (grey boxplots) and indirect exposure (black boxplots) of fluoxetine. i)- SOD activity response; ii)- CAT activity response; iii)- GST activity response; iv)- AChE activity response; v)- LPO level response; vi)- DNAd level response. Number of replicates for direct exposure: n=41 for CAT, SOD, GST and AChE; n=33 foı LPO and DNAd. Number of replicates for indirect exposure: n=62 for SOD, CAT, GST and AChE; n=47 for LPO and DNAd. Boxplots represent median and whiskers represent minimum and maximum values; lowercase letters indicate significant differences at p-value < 0.05 for Kruskal-Wallis test for the direct exposure and uppercase letters indicate significant differences at p-value < 0.05 for Kruskal-Wallis test for the indirect exposure; asterisks denote significant differences among the same treatments on the direct and indirect exposure trials for Mann-Whitney test.

Considering the attack test time and the turn-over test time performed for *C. maenas* (Fig. 4.3), no significant differences were found. Nevertheless, the medium and high treatments had the longest attack time responding to the contaminated water, in the direct exposure experiments. On the other hand, in the indirect exposure trials, the individuals from the high treatment had the shortest response to the food stimulus. Regarding the turn-over tests, in the direct exposure, the samples from the low treatment exhibited the longest period to turn-over, while in the indirect exposure, the samples from the control and high treatments had the longest periods.

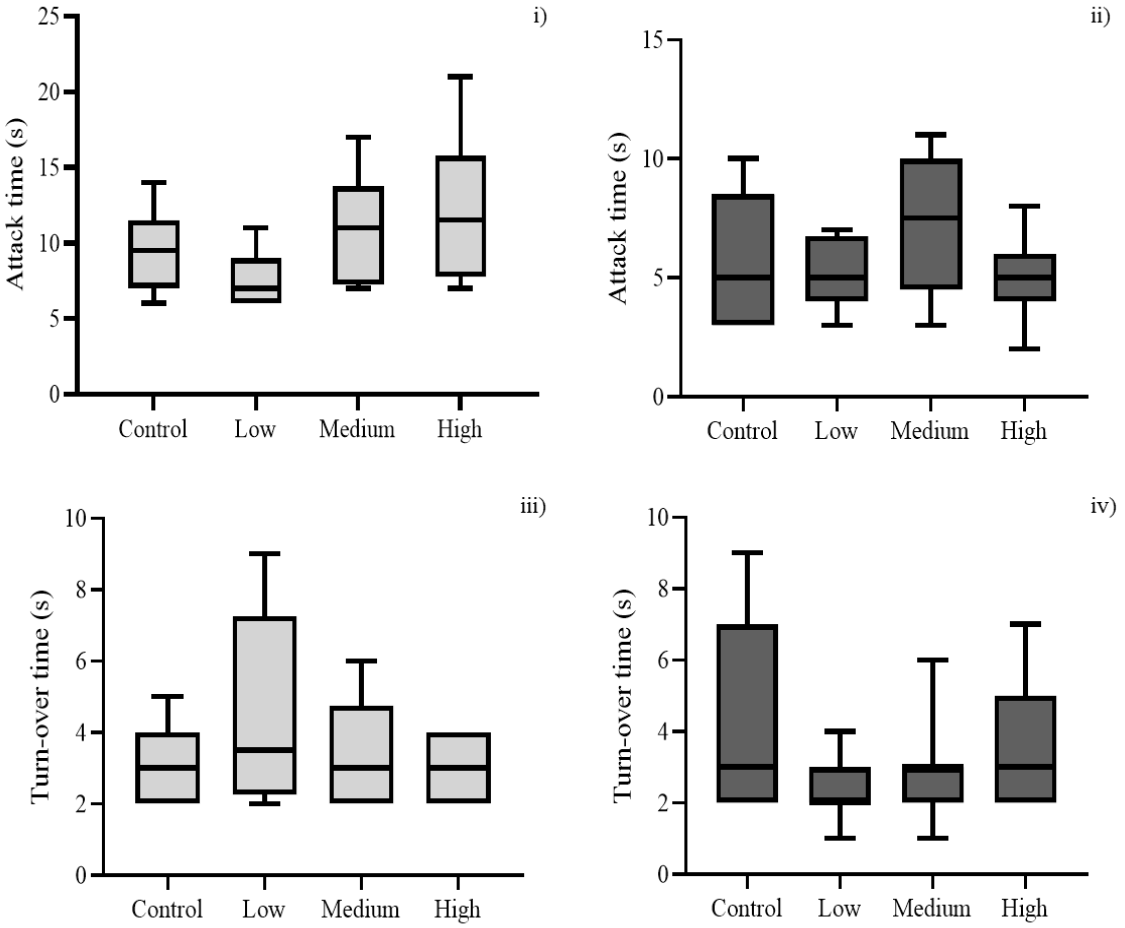

Fig. 4.3 - Attack test and turn-over test of *C. maenas*. The i) and iii) graphics correspond to the direct exposure trial, while the ii) and iv) graphics correspond to the indirect exposure trial. Number of replicates for the attack time: direct exposure, n=62; indirect exposure, n=62. Number of replicates for the turn-over time: direct exposure, n=62; indirect exposure, n=62. Boxplots represent median and whiskers represent minimum and maximum values.

4.3. Concentration-response analysis

To test the relationship between the applied fluoxetine concentration and the biomarkers responses as well as between biomarkers, a correlation analysis was performed. Regarding *P. serratus*, in the direct exposure trial (Table 4.1), positive correlations were found between DNAd and CAT and the exogenous fluoxetine dose applied (r_s= 0.549, ρ-value < 0.05, r_s= 0.370, ρ-value < 0.05, respectively), moreover negative correlations were found for GST and AChE comparative to treatments (r_s= - 0.366, ρ-value < 0.05, r_s= - 0.395, ρ-value < 0.05, respectively). On the other hand, for the indirect exposure experiment (Table 4.2), concerning the treatments correlations, a positive one was found for CAT (r_s= 0.535, ρ-value < 0.05), and a negative correlation was found for SOD (r_s= - 0.498, ρ-value < 0.05). Concerning the correlation analysis between biomarker responses, were just found two significant correlations on the indirect exposure, namely, a positive correlation between AChE and SOD

(r_s= 0.426, p-value <0.05) and a negative correlation between CAT and SOD (r_s= - 0.355, p-value <0.05, Table 4.2).

Comparatively to *C. maenas* correlation analysis, only in the indirect exposure were found significant correlations between treatments and biomarkers responses (Table 4.4), namely a negative correlation with DNAd and a positive correlation with GST (r_s= - 0.476, p-value <0.05 and r_s= 0.399, p-value <0.05, respectively). Regarding the correlations between biomarkers it was only found a negative significant correlation between CAT and LPO in the direct exposure individuals (r_s= - 0.544, p-value <0.05, Table 4.3).

Concerning the behavioural trials (attack time trial and turn-over time trial) no significant correlations were found.

Table 4.1 - Spearman correlation results of the direct exposure of *P. serratus* to fluoxetine. Asterisks represent significant differences.

		Fluoxetine concentration	*LPO*	*DNAd*	*CAT*	*SOD*	*GST*	*ACHE*
Fluoxetine concentration	Spearman Correlation	1	0.201	0.549*	0.370*	-0.068	-0.366*	-0.395*
	Level of significance		0.254	0.001*	0.15*	0.674	0.018*	0.009*
	N	43	34	33	43	41	41	43
LPO	Spearman Correlation		1	0.096	0.153	0.041	-0.252	-0.193
	Level of significance			0.597	0.386	0.822	0.157	0.274
	N		34	33	34	32	33	34
DNAD	Spearman Correlation			1	0.114	0.045	-0.179	-0.031
	Level of significance				0.526	0.809	0.328	0.862
	N			33	33	31	32	33
CAT	Spearman Correlation				1	0.183	0.114	-0.243
	Level of significance					0.252	0.478	0.116
	N				43	41	41	43
SOD	Spearman Correlation					1	0.117	0.143
	Level of significance						0.477	0.371
	N					41	39	41
GST	Spearman Correlation						1	0.068
	Level of significance							0.675
	N						41	41
ACHE	Spearman Correlation							1
	Level of significance							
	N							43

Table 4.2 - Spearman correlation results of the indirect exposure of *P. serratus* to fluoxetine. Asterisks represent significant differences.

		Fluoxetine concentration	*LPO*	*DNAd*	*CAT*	*SOD*	*GST*	*AChE*
Fluoxetine concentration	Spearman Correlation	1	0.001	-0.015	0.535*	-0.498*	-0.010	-0.067
	Level of significance		0.995	0.920	0.000*	0.000*	0.937	0.602
	N	63	47	47	62	62	61	62
LPO	Spearman Correlation		1	0.122	0.106	-0.179	0.159	-0.015
	Level of significance			0.413	0.483	0.234	0.28	0.919
	N		47	47	46	46	45	47
DNAd	Spearman Correlation			1	0.004	0.019	0.081	0.051
	Level of significance				0.977	0.901	0.598	0.736
	N			47	46	46	45	47
CAT	Spearman Correlation				1	-0.355*	-0.088	-0.192
	Level of significance					0.005*	0.503	0.139
	N				62	61	60	61
SOD	Spearman Correlation					1	0.067	0.426*
	Level of significance						0.609	0.001*
	N					62	60	61
GST	Spearman Correlation						1	0.120
	Level of significance							0.362
	N						61	60
ACHE	Spearman Correlation							1
	Level of significance							
	N							62

Table 4.3 - Spearman correlation results of the direct exposure of *C. maenas* to fluoxetine. Asterisks represent significant differences.

		Fluoxetine concentration	*LPO*	*DNAd*	*CAT*	*SOD*	*GST*	*AChE*
Fluoxetine concentration	Spearman Correlation	1	0.191	-0.051	-0.216	-0.245	0.106	-0.185
	Level of significance		0.296	0.780	0.243	0.176	0.564	0.327
	N	32	32	32	31	32	32	30
LPO	Spearman Correlation		1	-0.264	-0.544*	-0.139	0.080	0.179
	Level of significance			0.144	0.002*	0.449	0.662	0.344
	N		32	32	31	32	32	30
DNAd	Spearman Correlation			1	0.108	-0.019	-0.319	0.182
	Level of significance				0.564	0.918	0.075	0.336
	N			32	31	32	32	30
CAT	Spearman Correlation				1	0.168	0.273	-0.001
	Level of significance					0.367	0.137	0.994
	N				31	31	31	29
SOD	Spearman Correlation					1	0.076	0.036
	Level of significance						0.678	0.849
	N					32	32	30
GST	Spearman Correlation						1	0.114
	Level of significance							0.548
	N						32	30
ACHE	Spearman Correlation							1
	Level of significance							
	N							30

Table 4.4 - Spearman correlation results of the indirect exposure of *C. maenas* to fluoxetine. Asterisks represent significant differences.

		Fluoxetine concentration	LPO	DNAd	CAT	SOD	GST	AChE
Fluoxetine concentration	Spearman Correlation	1	0.157	-0.476*	0.314	-0.276	0.399*	0.315
	Level of significance		0.406	0.010*	0.110	0.133	0.32*	0.084
	N	31	30	28	27	31	29	31
LPO	Spearman Correlation		1	-0.072	0.182	0.042	0.171	0.075
	Level of significance			0.721	0.373	0.825	0.383	0.695
	N		30	27	26	30	28	30
DNAd	Spearman Correlation			1	-0.332	0.339	-0.039	0.024
	Level of significance				0.105	0.077	0.849	0.904
	N			28	25	28	26	28
CAT	Spearman Correlation				1	-0.013	-0.092	0.266
	Level of significance					0.947	0.663	0.180
	N				27	27	25	27
SOD	Spearman Correlation					1	-0.324	0.030
	Level of significance						0.087	0.872
	N					31	29	31
GST	Spearman Correlation						1	0.302
	Level of significance							0.111
	N						29	29
ACHE	Spearman Correlation							1
	Level of significance							
	N							31

4.4. Biomarker profile multivariate analysis

A close evaluation of the multivariate analysis applying CAP analysis, using both *P. serratus* and *C. maenas* biomarkers data, indicated a clear differentiation between the two species (Fig. 4.4-i), with a classification efficiency of 84.2 %. Moreover, a slight differentiation was noticed between exposures for *P. serratus*, using the considered biomarkers as biochemical descriptors of exposure to fluoxetine. The CAP plot using only the *P. serratus* individuals exposed to both types of exposure trials represented in Fig. 4.4-ii), evidence a separation of the *P. serratus* individuals from the direct and indirect exposure trials (classification efficiency of 68 %), indicating that the used biomarkers respond differently to the type of exposure applied, except for AChE that had a stronger relationship with the indirect exposure concentrations. Using the same approach and analysing the CAP analysis referent to the *C. maenas* individuals exposed directly and indirectly to fluoxetine (Fig. 4.4-iii) it is possible to observe a slight separation between the direct and indirect exposure, with the indirect exposure more associated to the biomarkers responses, except for GST, which was more related with the direct exposure, reaching a classification efficiency of 44.5 %, although the responses were more tangled compared to *P. serratus* individuals. This indicates that for *C. maenas* the tested biomarkers are more insensitive to the fluoxetine exposure form.

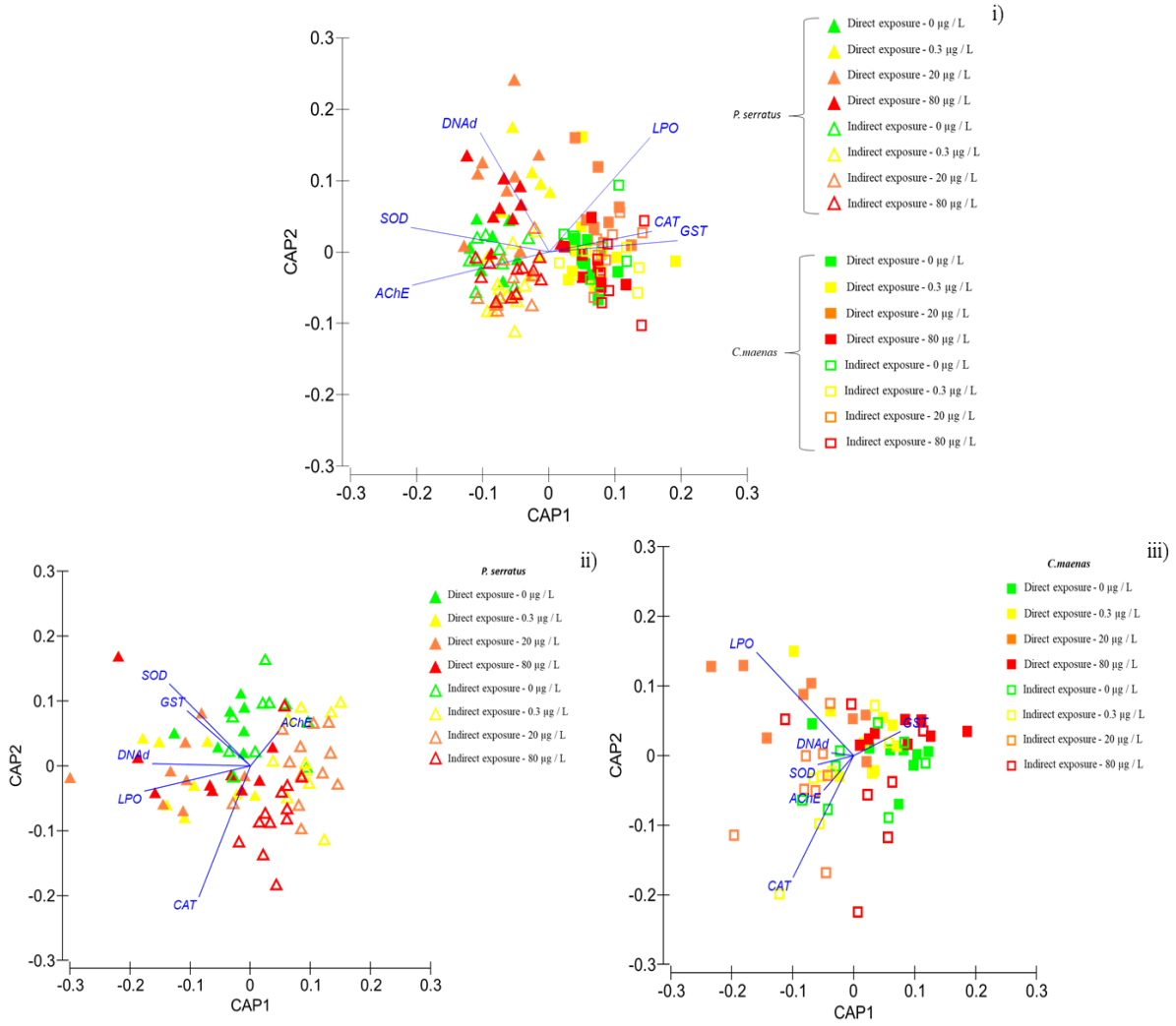

Fig. 4.4 - Canonical analysis plot based on *P. serratus* and *C. maenas* exposure trials to fluoxetine (i). Direct and indirect exposure trials and different fluoxetine treatments profile of *P. serratus* individuals (ii). Direct and indirect exposure trials and different fluoxetine treatments profile of *C. maenas* individuals (iii).

5. Discussion

Aiming to describe how the antidepressant fluoxetine interacts with estuarine species, namely in its uptake from the water, as well as, along a trophic segment of the Tejo estuary, both *P. serratus* and *C. maenas* were exposed to different fluoxetine concentrations and several biomarkers were analysed, namely the antioxidant and biotransformation enzymes, the neurotoxicity biomarker and lastly biomarkers of effect. This is of utmost importance to understand the impact of anti-depressants present in estuarine waters (Fonseca *et al.*, 2020) and its possible cascade of events throughout an estuarine trophic web. This pharmaceutical compound already showed to have significant effects at the first level of the trophic segment, impairing severely not only the primary productivity of a model estuarine diatom but also impacting its nutritional and energetic value, with evident shifts in the fatty acid composition of these primary producers (Feijão *et al*., 2020). Thus, and considering this, becomes important to address the impacts of this anti-depressant in the upper trophic levels including the planktivorous and carnivorous primary consumers (*P. serratus* and *C. maenas* respectively), as well as the impact of the different exposure forms (direct or indirect/trophic).

Antioxidant enzymes play a fundamental role in the defence mechanisms of the organisms to the increase of reactive oxygen species (ROS), due to the exposure and uptake of contaminants, such as fluoxetine (Van der Oost *et al*., 2003). For the direct exposure of *P. serratus* to fluoxetine, the increase of CAT activity suggests that fluoxetine overwhelmed the organism's first antioxidant defences. This results in deleterious effects on DNA and in lipid peroxidation increase, which was confirmed by the observed increase in the DNA damage and LPO levels in the medium and high fluoxetine treatments. Additionally, oxidative stress is an undeniable result of pharmaceutical toxicity, and the elevated levels of LPO and DNA damage indicate an oxidative stress condition in *P. serratus* organisms exposed to the highest fluoxetine concentrations tested. Moreover, the increase in LPO and DNA damage even under higher CAT activity indicates that the enzymatic antioxidant defences are not enough to prevent an oxidative burst inside the cells. This behaviour was also observed in fluoxetine-exposed fish, with high CAT activity levels and simultaneous LPO and DNA damage increase in liver tissue (Duarte *et al*., 2020). Moreover, this antidepressant also showed to be able to induce oxidative stress in invertebrates, such as the clam *Corbicula fluminea* and the mussel *Mytilus galloprovincialis* (Chen *et al.*, 2015; Gonzalez-Rey *et al.*, 2013). On the other hand, the individuals exposed to fluoxetine by trophic means, CAT activity increased significantly only at the highest concentration of fluoxetine tested. This indicates that direct exposure triggers higher oxidative feedback than the indirect exposure. This is also corroborated by the lower levels of LPO and DNA damage. Comparatively to the direct exposure of *P. serratus*, the high activity of antioxidant enzyme CAT indicates a higher need to increase the defence mechanisms to metabolize the input of fluoxetine, despite SOD activity has not increased. The higher levels of LPO and DNA damage could also suggest that the primary defence systems were not able to fully neutralized the excessive ROS accumulation generated by fluoxetine input, therefore inducing oxidative damage in the organisms subject to direct exposure of this antidepressant. Similar effects on SOD and CAT antioxidant defences were obtained for the anti-inflammatory ibuprofen in mussels gills (Gonzalez-Rey and Bebianno, 2011). On the other hand, SOD enzyme revealed a decreased activity in the indirect exposure trial, opposing to the response observed in CAT enzyme activity, is also evidenced by the negative correlation between SOD and CAT. Normally, the activity of SOD generates H_2O_2 that is counterbalanced and detoxified by CAT activity, that converts hydrogen peroxide into less reactive components, water and molecular oxygen. Hence, these results can suggest the generated H_2O_2 does not result from the SOD activity but probably due to the direct interaction from fluoxetine interactions in the different cellular components. On the other hand, previous reports show that under severe stress, SOD activity can be impaired, due to excessive ROS accumulation and direct enzyme and tissue injury, as previously observed for rat liver and crustaceans exposed to fluoxetine by

Djordjevic *et al.* (2011) and Ding *et al.* (2017), respectively. Additionally, Milan *et al.* (2013), reported downregulation of SOD activity in the clam digestive gland exposed to ibuprofen. Moreover, in the indirect exposure trials, a positive significant correlation between SOD and AChE activities was found, supporting the effective defence of the antioxidant enzymes SOD and CAT, even though no significative differences were found for AChE enzyme activity. Regarding the direct exposure, the stated decrease of AChE activity, suggests a possible increase of fluoxetine neurotoxicity on *P. serratus* motor functions. Moreover, Ding *et al.* (2017), reported that AChE activity in crustacean *Daphnia magna* could be inhibited by fluoxetine, as well as Munari *et al*. (2014) stated that the clam *Venerupis philippinarum* suffered an AChE activity decreased in gill when exposed to 1 and 5 µg L^{-1} of fluoxetine, highlighting thus, the neurotoxicity of the pharmaceutical fluoxetine on aquatic invertebrates.

The biotransformation enzyme GST helps to prevent the effect of ROS, promoting the metabolization of xenobiotic compounds and facilitating its excretion, by catalysing the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates. In the indirect exposure, the enzyme GST, revealed a decrease in activity in the low and medium treatments, returning to control values in the high treatment, indicating that this enzyme follows a hermetic response model, as defined by Calabrese and Baldwin (2001). In this case, there is an inhibitory response at low dosages, followed by an induction response at higher dosages, resulting in a U-shaped curve, instead of the typical dose-response linear correlation. Comparing to direct exposure trial, in general, GST activity showed a tendency to decrease with the increase of fluoxetine exposure concentrations. The inhibition of GST activity at higher concentrations may be due to the less GSH available to stimulate the detoxifying process, as reported in previous studies. For instance, Duarte *et al.* (2019), observed that fluoxetine increased GST activity up to 10 µg L^{-1} in fish liver; Franzellitti *et al*. (2014), described that GST activity was significantly increased in marine mussels' digestive gland at 0.3 ng L^{-1}, and at the higher concentration levels in marine mussels' gills. Moreover, a similar induction of GST enzymatic activity has been reported for other compounds. The exposure of *Ruditapes philippinarum* to caffeine, ibuprofen, carbamazepine and novobiocin (0.1, 1, 5, 10, 15, and 50 mg L^{-1}) reported an increase of biotransformation enzyme GST activity (Aguirre-Martínez *et al.*, 2016). Overall, *P. serratus* showed higher susceptibility to the antidepressant fluoxetine in the direct exposure trial.

Moving on to an upper trophic level, the shrimp-eating crab *C. maen*as was evaluated concerning its direct and trophic (feeding on contaminated *P. serratus*) exposure to fluoxetine. Concerning *C. maenas* exposed directly to fluoxetine, its CAT activity had a significant decrease in both low and high treatments, whereas in the medium treatment, the values were similar to the control. The same results appear to occur with the opposite similarity for low and high treatments on LPO levels, suggesting a failure in the antioxidant defence system. However, Rodrigues *et al*. (2014) and Lee *et al.* (2013) demonstrated contradictory results in individuals exposed to the antidepressants. These authors suggest that both in humans as in *C. maenas* individuals, antidepressants decrease ROS production, thus, decreasing LPO levels, and subsequently enhancing CAT activity. Nevertheless, the negative correlation found between CAT and LPO may suggest that fluoxetine had increased the overproduction of reactive oxygen species resulting in oxidative stress, hence increasing levels of LPO, especially in the low and high treatments, that weren't able to be reduced by the antioxidant defence enzyme CAT, as well as by SOD enzyme, that did not have significant differences. Contrary, in the indirect exposure trials, *C. maenas* CAT activity showed a bell-shape form curve with higher enzymatic activity induced at low and medium treatments, followed by inhibition at the higher treatment. Calabrese and Baldwin (2003) suggested that this process can result from a mechanism of action able to induce effects at low concentrations. Regarding SOD activity, it is known that it is the primary defence to the increase of ROS by xenobiotic exposure (der Oost *et al.*, 2003). For both exposure trials, similar SOD enzymatic activities indicate that no significant oxidative stress is induced with the increasing fluoxetine concentration. Ding *et al.* (2017) also showed that there were no significant changes in SOD activity in

the crustacean *Daphnia magna*, exposed to the antidepressant fluoxetine. On the other hand, Byeon *et al*. (2020) demonstrated that oxidative damage may occur in rotifer *Brachionus koreanus*, alongside a concomitant increase in the SOD activity, indicating a need to activity counteractive measures. Also, Chen *et al.* (2015) showed evidence that SOD activity in the gills and digestive glands of *Corbicula fluminea* clam significantly decreased when subjected to 5 µg L^{-1} and 50 µg L^{-1} concentration of fluoxetine.

The activity of the biotransformation enzyme GST, increased in the individuals exposed to the highest concentration of fluoxetine throughout trophic exposure, suggesting that GST activity could have been induced to counteract fluoxetine effects at that particular target concentration, indicating that this may be a possible threshold. Likewise, Mesquita *et al*. (2011) evidence the same effect in the biotransformation enzyme GST, with the increase of GST activity at the highest levels of exposure of fluoxetine. Superoxide dismutase catalyses the conversion of superoxide radicals to oxygen and hydrogen peroxide, which is then metabolized by several peroxidases and by GST, promoting the reduction of hydrogen peroxide. The negative correlation between SOD and GST can indicate that hydrogen peroxide is not only being produced by SOD antioxidant activity, but also by direct Fenton reaction thus, inducing an over overcompensation by GST enzyme, preventing the inactivation of SOD as H_2O_2 in excess can act as an inhibitor of SOD (Casano *et al*., 1997).

Previous studies showed evident effects on *C. maenas* locomotion associated with the increase of AChE enzyme activity (Mesquita *et al*., 2011). In the present study, no locomotion inhibition nor behaviour effects were observed. Alongside AChE activity levels were unaffected in both trials, indicating that no neurotoxicity was induced under fluoxetine exposure. Additionally, despite the significant decrease of anxiety-like behaviour when subject to high concentrations of fluoxetine, Hamilton *et al*. (2016) concluded that fluoxetine had no impact on the mobility or aggression of shore crab, *Pachygrapsus crassipes*. On the other hand, other crabs, molluscs and fishes, exhibited behaviour and anxiety variations when subjected to target levels of fluoxetine and other pharmaceuticals (Milan *et al*., 2013; Munari *et al*., 2014; Park *et al*., 2012).

The failure of the antioxidant enzymes defences to prevent the excess of ROS production can lead to oxidative damage, including enzyme inhibition, lipid peroxidation, DNA damage that ultimately, can lead to organism failure and subsequently death (der Oost *et al*., 2003). In the present study the significative increase of LPO levels, in the low and high treatments of the direct exposure trial of *C. maenas* to fluoxetine, indicate that crabs were under oxidative stress. This could be supported by the negative correlation between LPO levels and CAT activity, as mentioned above. Duarte *et al.* (2020) demonstrated that exposure to 3 µg L^{-1} of fluoxetine can inhibit the detoxification processes, thus increasing lipid peroxidation and DNA damage in meagre. Also, malondialdehyde (MDA) levels, a marker of lipid peroxidation, were significantly increased in the higher concentration groups in response to the antibacterial florfenicol exposure of crab *Portunus trituberculatus* (Ren *et al*., 2017). Lastly, even though there were no significant differences in both trials for DNAd levels, there is a tendency, for the decrease in DNA damage levels along with the increasing concentration of fluoxetine, in the trophic exposure. This can be due to the scavenging CAT activity, since ROS are the major cause of DNA damage in invertebrates (Dong *et al*., 2012). Overall, C. *maenas* did not show a significant susceptibility to the antidepressant fluoxetine in both exposure trials.

In this study, the biomarkers responses patterns in general, and also, corroborate by the multivariate analysis, disentangled both species from each other regarding their responses to the antidepressant fluoxetine, suggesting that *P. serratus* had a more significative relation in concentration-response to the exposure to fluoxetine, and experience more deleterious effects comparing to *C. maenas*. Furthermore, comparing the direct and indirect exposure to this pharmaceutical, there was a greater differentiation between the results in *P. serratus* relative to *C. maenas*. This suggests that *C. maenas* could be less susceptible to fluoxetine either by direct or indirect exposure. Considering the application

of the tested biomarkers as potential descriptors for the evaluation of *P. serratus* and *C. maenas* exposure to fluoxetine, these appear to be efficient biomarkers of the exposure type, highlighting the abovementioned differences between the exposure trials here reported.

6. Conclusion

Estuarine areas are characterized by the surrounding discharges of human waste. The percentage of pharmaceutical waste has been increasing, and thus, the ecological studies have shifted its aim to encompass these new emergent contaminants. Therefore, the importance of knowing how pharmaceuticals, such as the antidepressants, impact these environments is vital to promote a regulatory waste discharge. Understanding how fluoxetine interacts with the marine and estuarine organisms, namely how performs in the direct uptake of fluoxetine from the water into the organisms, besides with the uptake along the trophic chain, can draw the steps needed to take to achieve a sustainable estuarine environment.

Fluoxetine acts as a selective serotonin reuptake inhibitor increasing serotonergic neurotransmission at organism synapses. Potential stress effects of fluoxetine were measured using a set of biomarkers for invertebrate health status that included antioxidant enzyme activities, acetylcholinesterase activity, lipid peroxidation and DNA damage. Furthermore, fluoxetine levels in the two species and water were supposed to be measured to assess concentration-dependent relationships between the observed biological effects and the bioaccumulation potential of the pharmaceutical, yet due to logistic complications during the Covid-19 pandemic, these last measurements were not performed. Nevertheless, these procedures are important to complement the present work and should be made in future research.

According to literature, there are few studies addressing fluoxetine effects in *P. serratus* and *C. maenas*, in this sense our study gives additional knowledge to this field. To the best of our knowledge, no other studies report the effects of direct and indirect exposure to a pharmaceutical, simulating the contamination of fluoxetine that occurs along a trophic segment in an estuary.

Regarding *P. serratus* we observed a higher sensitivity to fluoxetine exposure, namely in the direct exposure which appears to induce more deleterious effects on *P. serratus,* presenting a higher degree of oxidative stress under direct exposure when compared to the trophic intake of fluoxetine. Concerning *C. maenas* there was no great differentiation among biomarkers responses comparing both exposure trials, although some separation could be detected when the whole set of biomarkers is used to disentangle the exposure groups. Overall, with this study, we can conclude that *P. serratus* showed higher susceptibility to the antidepressant fluoxetine and that *C. maenas* could be more resistant to this pharmaceutical.

Despite these results, several ways to complement this study undergo by performing bioaccumulation and bioconcentration studies, which are a fundamental key to acknowledge how fluoxetine affects the organisms. Nevertheless, water chemical analysis can also be a path to understand the potential toxicity bioaccumulation effects on the organisms. Likewise, knowledge of pharmaceutical contamination and its biological effects at higher levels of the trophic chain is essential to tackling the potential impacts of pharmaceuticals within a more significant ecological framework.

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